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Interactions between Dermatophyte Fungi and Staphylococci or Brevibacterium *in Vitro*

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Interactions between dermatophyte fungi and staphylococci or brevibacteria on a new skin-based culture medium are described. Penicillin production by the fungus selects penicillin resistant *S. aureus* or *B. epidermidis*. Fungi are inhibited by brevibacteria but not by the staphylococci. "Keratolysis" by fungi may contribute to the growth nutrients of staphylococci.

Previous studies [1,2] have shown that dermatophyte fungi produce a variety of antibiotics including penicillin-like and streptomycin-like substances and that these are produced *in vivo* where they bring about changes in the bacterial flora of dermatophyte induced lesions. Youssef and her colleagues [2] reported that, in an *in vitro* model, dermatophyte fungi would select penicillin-resistant cocci from a mixture of resistant and sensitive cells. These studies are in agreement with studies by Bibel and Le Brun [3] and Bibel and Smiljanic [4]. This paper reports further studies on interaction between the fungi and bacteria.

MATERIALS AND METHODS

Strains Used

Trichophyton mentagrophytes Tm 7 of Youssef series, produces penicillin but no other antibiotics detectable by use of *Bacillus subtilis* (NCTC 8236) or the Oxford staphylococcus. Strain Tm 1325 produces no detectable antibiotic using these same test organisms.

Staphylococcus aureus NCTC 8325; 8325 N sensitive to antibiotics and does not contain plasmids. 8325 pen resistant to penicillin and erythromycin and contains only the PI258 plasmid mediating these resistances.

Brevibacterium epidermidis species nova [5]. Three isolates were used with domestic code numbers L1, P159, D731. Strain L1 has a Minimum Inhibitory Concentration for penicillin of about 2.5 units/ml and the other 2 strains have Minimum Inhibitory Concentration of about 0.5 units/ml. These strains do not produce penicillinase (beta-lactamase).

Inhibition of Bacteria by Fungi

The synthetic skin medium (SSM) of Ryall, Holt and Noble [6] was used. This consists of synthetic sweat solution and human callus stratum corneum as sole nutrients solidified with agar. On this medium dermatophyte fungi release antibiotics with maximum production at the edge of the fungal colony. There is a zone of keratolysis extending beyond the colony edge. This is illustrated digrammatically in Fig 1. Fungi growing on this medium more closely resemble fungi in skin in their microscopic morphology than do fungi grown on wholly artificial media.

Dermatophyte fungi were point inoculated at the center of the medium in 90 mm Petri dishes and incubated at 31°C for 24 days to give colonies of about 45 mm diameter. The agar was then inverted to expose the uninoculated surface. This was seeded with bacteria as single pure cultures or as mixtures of strains at a cell density of about 2.5×10^3 colony forming units per mm^2. The plates were then incubated at 33°C and examined after 24, 48, 72 hr (and 168 hr when *Brevibacterium* was used). Duplicate plugs 5 mm in diameter were cut from the agar using a sterile cork borer; the sites chosen were the center of the underlying fungal colony, the edge of the colony, within the zone of keratolysis surrounding the edge of the colony and in the unkeratolysed zone at the margin of the petri dish. Plugs were shaken in 9 ml of chilled quarter strength Ringers solution with 6 mm glass beads for 10 min, serial dilutions of the suspensions were made and inoculated onto nutrient agar (Oxoid Blood agar base CM 55) or onto nutrient agar containing 15 μg/ml cadmium nitrate. Colonies were counted after 24 h incubation at 37°C. The standard error of the mean between the duplicate samples was of the order of 12% of the mean count. Cadmium nitrate was used to select the penicillin resistant variants of *S. aureus* since the PI258 plasmid also bears genes governing resistance to cadmium (MIC >15 μg/ml) while the sensitive strain is inhibited by less than 5 μg/ml cadmium nitrate. In a study of the growth of the strain 8325 pen on plain and cadmium containing agar, no difference could be detected in the number of viable units which gave rise to colonies (t=0.3, n=26 pairs, $P > 0.10$).

Inhibition of Fungi by Bacteria

Colonies of *T. mentagrophytes* Tm 7 were obtained by point inoculating SSM plates and incubating for 6–8 days. Half of the remaining agar surface bearing the fungal colony was then inoculated with a suspension of bacteria using cultures of 10^7 cfu/ml. Mixed cultures were then incubated at 33°C for 28 days, bacterial growth being visible after 18 hr.

RESULTS

Inhibition of Bacteria by Fungi

Results are shown in Tables I and II as the logarithm of the percentage viable count with 0 hour (0h) as 100% = 2.00.

Table I shows that, as expected, the presence of penicillin especially at the edge of the colony, suppresses the growth of the penicillin sensitive, but not the resistant strain of *S. aureus*. This is illustrated for the colony edge data only in Fig 2. In mixed culture, penicillinase production by the resistant strain results in partial growth of the sensitive variety; however, in the absence of penicillin the sensitive variety outgrows the resistant one in mixed culture.

Table II shows that *Brevibacterium* strain L1 was strongly inhibited at the colony edge (illustrated in Fig 3), but that the other 2 strains were less strongly inhibited at the colony edge and were not inhibited at other sites on the agar surface. Levels of about 3 units/ml of penicillin are produced at the colony edge.

Inhibition of Fungi by Bacteria

After 18 hr incubation, penicillin resistant Staphylococci and strains of *B. epidermidis* P159 and D731 had grown up to the colony edge but the sensitive *S. aureus* and *Brevibacterium* L1 were inhibited in a zone 2–4 mm from the colony edge. This is consistent with the results described in the previous section.

After a further 28 days incubation, fungal hyphae had grown into the area occupied by both the staphylococci although at a reduced rate compared with the uninoculated half of the agar. However plates on which brevibacteria had been inoculated showed complete inhibition of fungal growth by strain L1 and strong inhibition by the other 2 strains. Indeed by 28 days the growth of the brevibacteria had extended into the zone occupied

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Abbreviations:
MIC: minimum inhibitory concentration

TABLE I. *Growth of penicillin sensitive and resistant* S. aureus *in the presence of penicillin producing and non-producing strains of* Trichophyton mentagrophytes

Time	Nonproducer fungus Tm 1325				Producer fungus Tm 7			
	Single cultures		Mixed cultures		Single cultures		Mixed cultures	
	S[a]	R[a]	S	R	S	R	S	R
0 h	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
24 h C[b]	4.29	4.04	4.60	4.31	0.18	4.41	1.61	5.06
E[b]	4.80	4.66	4.94	4.39	<0.01	4.19	<0.01	4.07
K[b]	4.38	4.91	4.88	4.87	2.61	3.66	2.04	3.25
U[b]	3.34	4.12	3.97	4.08	2.40	2.70	1.05	2.61
48 h C	5.44	5.33	6.14	5.15	<0.01	5.65	3.35	5.95
E	5.86	5.49	5.57	4.61	<0.01	5.70	2.08	6.25
K	5.35	5.49	5.23	4.89	4.92	5.43	3.14	4.70
U	4.01	4.36	4.30	4.26	4.57	5.24	2.92	4.40
72 h C	5.56	5.75	5.97	4.74	<0.01	5.80	3.17	6.00
E	5.63	5.95	6.05	5.13	<0.01	5.82	2.42	6.29
K	5.43	5.68	5.54	4.41	5.41	5.68	3.56	5.19
U	4.58	4.90	5.06	4.37	5.38	5.59	3.56	4.67

Data presented as log percentage viable units with 0 h as 100% = 2.00.
[a] S = sensitive and R = resistant to penicillin.
[b] C = Center of fungal colony, E = edge of colony, K = keratolysed zone, U = unkeratolysed zone as indicated in Fig 1.

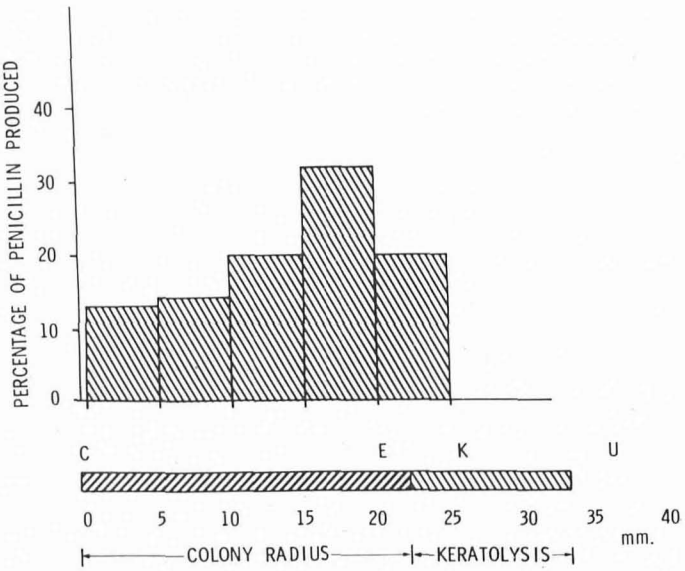

FIG 1. Distribution of penicillin in growth medium in relation to dermatophyte colony radius. *C* = center of colony, *E* = edge of colony, *K* = keratolysed zone, *U* = unkeratolysed zone of agar.

TABLE II. *Growth of various strains of* Brevibacterium epidermidis *in the presence of penicillin producing and nonproducing strains of* Trichophyton mentagrophytes

Time	Nonproducer fungus			Producer fungus		
	L1	P159	D731	L1	P159	D731
0 h	2.00	2.00	2.00	2.00	2.00	2.00
24 h C[b]	4.04	4.61	4.77	1.05	4.59	4.74
E[b]	4.12	4.80	4.99	−0.08	1.64	3.33
K[b]	3.30	3.62	4.02	1.30	4.85	4.61
U[b]	3.47	3.61	3.60	1.30	5.37	4.97
48 h C	5.50	6.46	6.90	0.14	6.23	6.38
E	5.83	6.49	3.76	0.18	3.45	4.56
K	4.93	6.46	6.61	0.36	3.17	5.16
U	4.69	5.61	5.74	1.92	6.45	6.32
72 h C	6.42	6.75	7.41	1.86	7.19	6.60
E	6.62	6.91	7.30	−0.44	4.95	4.90
K	6.06	6.39	6.91	2.36	5.95	6.61
U	5.35	6.32	6.49	5.18	6.66	5.85
168 h C	8.00	7.50	8.20	1.81	5.29	8.03
E	7.89	7.60	8.25	<0.01	5.49	7.88
K	7.68	6.97	7.28	1.04	6.25	7.28
U	7.54	6.32	6.48	5.33	6.47	5.73

Data presented as log percentage viable units with 0 h = 100% = 2.00.
[b] C = center of colony, E = edge of colony, K = keratolysed zone, U = unkeratolysed zone as indicated in Fig 1.

by the fungi. Although this inhibition was readily seen on SSM agar it could not be reproduced on nutrient agar, peptone yeast-extract agar or Sabouraud's agar.

DISCUSSION

The studies on inhibition of staphylococci by fungi confirm experience that penicillin production by the fungi will select for penicillin resistance in mixed populations of bacteria although penicillinase production by the resistant cells will permit restricted growth of the sensitive cells.

Also of interest is the demonstration of the "penalty" of possessing a plasmid in the absence of a selective agent, an effect that can usually only be demonstrated in mixed cultures [7,8]. It is clear that SSM is not a fully nutrient medium for *S. aureus* for the "penalty" effect is only expressed in restricted growth conditions (Jay Naidoo, personal communication). It is evident that *S. aureus* grows to higher densities in the keratolysed portion of the agar than in the unkeratolysed (mean log density keratolysed area 5.09, mean unkeratolysed area 4.28, n=12 pairs, t=4.5, $P < 0.001$). This seems likely to be the result of the liberation of nutrients from the keratin. The difference is less marked with the *Brevibacterium* species which are normal inhabitants of skin (mean log densities 5.85 and 5.43, n=12 pairs, t=0.71, $P > 0.10$).

Studies on *Brevibacterium* reported here (Table 2) show that another, and previously unsuspected, inhibitory agent is produced by the fungi. Previous experience had shown that, when tested with *Bacillus subtilis* or *S. aureus*, penicillinase would destroy all inhibitory activity, further the Brevibacteria tested were all resistant to penicillin by conventional tests. It is not known whether the substance which caused inhibition of L1 is a true antibiotic or some other metabolite.

The substance produced by *Brevibacterium* which inhibits the fungi is gaseous in nature. Experiments not reported in detail here show that inhibition continues at a lower level even when the 2 organisms are separated physically. *Brevibacterium* produce the gas methane thiol (CH_3SH)[9] which is toxic to fungi. We may speculate that keratolysis of stratum corneum by the fungi releases the L methionine needed as a substrate; this could explain why inhibition was not found when the organisms were grown on common laboratory media which may not contain sufficient sulfur-amino acids. Carbon dioxide, which has also been shown to inhibit dermatophyte fungi [10] would be

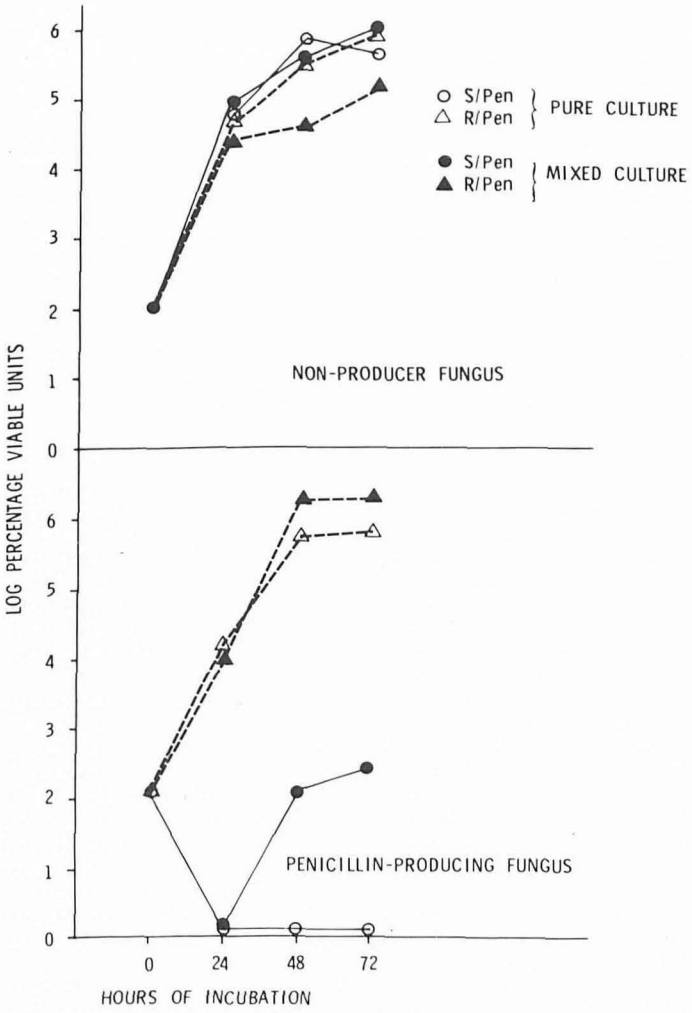

FIG 2. Interaction between dermatophyte fungi and *S. aureus* resistant and sensitive to penicillin. Colony edge data only. Growth rates are given as $\log_{10}$ percentage growth in relation to zero hours.

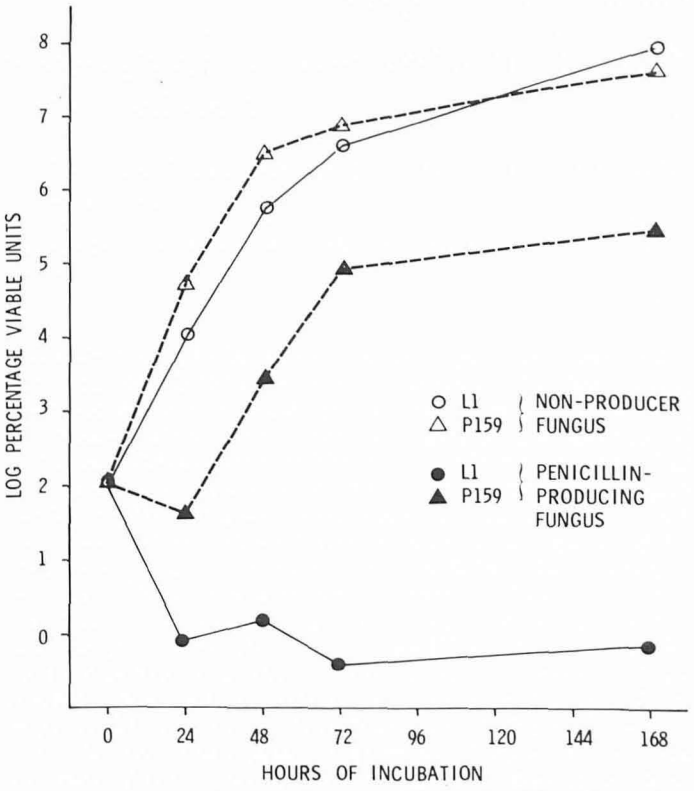

FIG 3. Interaction between dermatophyte fungi and *Brevibacterium epidermidis*, Colony edge data only. Growth rates are given as $\log_{10}$ percentage growth in relation to zero hours.

expected to be evolved on any medium.

Some of the complexity of fungal/bacterial interaction which must occur in tinea pedis [11] can be studied using the techniques reported here. However much remains to be done. This may require the development of an animal (or human?) model, since in the natural environment nutrient is supplied, and end products are removed, continuously. In common with others [12,13] we have shown that penicillin is produced in the epidermis of animals infected with dermatophytes but the models so far available to us do not seem sufficiently reproducible or predictable to embark on studies of interaction.

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